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(54) **Isolation and sequencing of the hazel FAd2-N gene**

(57) The invention relates to the isolation from hazel (*Corylus avellana* L.) of the FAD2-N gene coding for the $\Delta 12$ desaturase enzyme of the microsomal fraction and, in particular, provides the nucleotide sequence and the deduced amino-acid sequence of the gene and provides for its use as a probe for the isolation of other plant desaturases. It also relates to the use of this gene for altering the desaturase levels and consequently the fatty-acid composition of the plant.

EP 0 794 250 A1

Description

The present invention relates to the isolation from hazel (*Corylus avellana* L.) of the FAD2-N gene which codes for the $\Delta 12$ desaturase enzyme of the microsomal fraction.

More particularly, the invention relates to the nucleotide sequence, to the derived amino-acid sequence of the gene, and to its use as a probe for the isolation of other plant desaturases. It also relates to the use of this gene for altering the desaturase levels, and consequently the fatty-acid composition of the plant.

Alteration of the fatty-acid composition may have various applications in the industrial field. One of the greatest problems with hazelnuts is that they become rancid by oxidation. This is due to the auto-oxidation of unsaturated lipids with the consequent formation of volatile substances with a rancid odour which cannot easily be eliminated by the usual preservation systems. Amongst the possible strategies for reducing the tendency to become rancid, the best seems to be that of reducing the degree of unsaturation of the fatty acids present in the kernel oil, since susceptibility to auto-oxidation is positively correlated with this parameter. In fact, the rate of peroxide formation is correlated with the number of C=C double bonds in the fatty acids. The rate of auto-oxidation of the fatty acids in comparison with the oleate (18:1) is about 30 times greater in the linoleate (18:2) and 80 times greater in the linolenate (18:3). Moreover, the volatile substances resulting from the degradation of the linoleate and of the linolenate have a lower threshold of perception than those derived from the oleate. A reduction in linoleic acid should reduce the availability of substrates for lipoxygenase, reduce the loss of vitamin E during preservation, and reduce the production of volatile substances such as hexanals.

In the angiosperms, most of the synthesis of polyunsaturated lipids takes place by means of a single enzyme, that is, $\Delta 12$ (or $\omega 6$) desaturase (18:1 desaturase), of the endoplasmic reticulum, although there is an 18:1 chloroplast desaturase in the leaves of some plants. Moreover, this enzyme is responsible for more than 90% of the synthesis of polyunsaturated fatty acids in non-photosynthetic tissues such as, for example, in the kernels. The conversion of oleic acid (18:1) to linoleic acid (18:2) thus takes place by means of $\Delta 12$ desaturase, and from linoleic acid to linolenic acid (18:3) by means of $\Delta 15$ (or $\omega 3$) desaturase.

It has been shown with mutants of *Arabidopsis* that the FAD2 locus contains a gene which codes for the oleate desaturase enzyme of the endoplasmic reticulum (Okuley et al, 1994, The Plant Cell 6, 147-158). The FAD2 gene was in fact able to complement mutants of *Acabidopsis* which were deficient in desaturase activity of the endoplasmic reticulum. The gene coding for the same enzyme in soya has also recently been isolated and sequenced (Heppard et al, 1995, Plant Physiol., in press).

A reduction in the $\Delta 12$ desaturase levels should therefore lead to a reduction in the linoleic acid content and, as a secondary effect, probably also to a reduction in linolenic acid. In hazelnuts the percentage of linoleic acid varies from 5 to 15%; the percentage of linolenic acid is from 0.1 to 0.2%. A reduction in these fatty acids should therefore be useful in the preservation of hazelnuts. There is therefore clearly a need to isolate the gene which codes for the $\Delta 12$ desaturase of the endoplasmic reticulum. The sequence of the gene could thus be used for gene inactivation in hazelnut kernels. This inactivation could be carried out either by the antisense technique (Smith et al. (1988) Nature 334, 724-726) or by the "transwitch" technique (Flavell (1994) Proc. Natl. Acad. Sci. USA 91, 3490-3496). In the antisense technique, the hazel would have to be transformed by the entire FAD2-N gene or by portions thereof, inserted in the opposite direction to the regulating sequences. In the "transwitch" technique, the hazel would have to be transformed by an identical copy of the FAD2-N gene.

The subjects of the present invention are defined by the following claims.

Embodiments of the present invention will now be described with reference to the following drawings, in which:

Figure 1 shows the restriction map of the N2 genome clone,

Figure 2 shows the nucleotide sequence of the hazel FAD2-N gene; the amino-acid sequence of the coding portion is also shown;

Figure 3 shows the nucleotide sequence of the "I" clone of cDNA,

Figure 4 shows a comparison between the nucleotide sequences of the "I" and "N2" clones,

Figure 5 shows a comparison between the amino-acids of the "N2" gene and $\Delta 12$ desaturases of *Arabidopsis* and of soya,

Figure 6 shows the homology between hazel $\Delta 12$ desaturase and various desaturases of other plants both plastid and of the endoplasmic reticulum,

Figure 7 shows the expression of the N2 gene in various varieties of hazel both in the leaves and in the kernels.

Isolation and cloning of the FAD2 gene of *Arabidopsis thaliana* for use as a probe

In order to isolate the gene which codes for hazel $\Delta 12$ desaturase enzyme, it was necessary to use the FAD2 gene of *Arabidopsis* as a probe.

5 In order to isolate the *Arabidopsis* gene, two oligonucleotides were used as "primers" for the amplification of the sequences included between the start and the end of the gene. The oligonucleotides used were NOCC1 (CTGAATTC-CAGGTGGAAGAATGCC) which contains the Eco RI restriction site and the sequences corresponding to the portion between bases 100 and 116 of the gene (Okuley J. et al, 1994, The Plant Cell 6, 147-158) and NOCC4 (AGGAATTC-GACAATTTCTTCACCATCATGC) which contains the restriction site of the Eco RI enzyme and the sequences complementary to the portion between base 1245 and base 1266. The amplification reaction was as follows: 12.8 μ l H₂O, 2.5 μ l 10 x PCR buffer (Perkin Elmer), 2.5 μ l *Arabidopsis* genome DNA (10 ng/l), 1 μ l dNTP, each 2.5 mM, 2 μ l 25 mM MgCl₂, 1 μ l NOCC1 oligonucleotide (50 ng/ μ l), 1 μ l NOCC4 oligonucleotide (50 ng/ μ l) 0.2 μ l Taq I DNA polymerase (Perkin Elmer) (5 U/ μ l). The mixture thus prepared was subjected to 1 denaturing cycle for 1 minute at 94°C and to 40 cycles composed as follows: 30 seconds at 94°C, 1 minute at 52°C, 2 minutes at 72°C. The amplification products were separated on 1% agarose gel in TAE buffer (0.04 M Tris-acetate, 0.002 M EDTA) and stained with ethidium bromide at a concentration of 0.5 μ g/ml. The portion of gel containing the fragment of the expected length was withdrawn. In order to extract the DNA, 10 μ l of Qiaex resin (Qiaex extraction kit, firm Qiagen) were added for each 200 mg of gel. The supplier's method was then followed. The DNA was then supplemented with a tenth of a volume of 10X buffer (Boehringer) and 20 units of Eco RI enzyme (Boehringer). After incubation overnight at 37°C, the DNA was precipitated with 0.1 volumes of 5 M NH₄OAc and one volume of isopropanol. After 10 minutes at ambient temperature, the DNA was centrifuged for 20 minutes at 14000 rpm and the precipitate was washed with 70% ethanol. The DNA was resuspended in 15 μ l of H₂O. The concentration was determined on gel by comparison with a known standard.

The amplified fragment was inserted in the pUC18 vector. A ligation mixture was prepared as follows: 1 μ l pUC18 plasmid DNA cut with Eco RI (20 ng), 1.5 μ l fragment amplified with NOCC1 and 4 (25 ng), 1 μ l 10X ligase buffer (Boehringer), 1 μ l T4 DNA ligase (1 U/ μ l) (Boehringer), 4.5 μ l H₂O. The reaction mixture was incubated at 14°C for 12 hours.

In order to prepare competent cells, the method based on the compound hexamino-cobalt chloride was used (Maniatis, Molecular cloning, 1989, Cold Spring Harbor Laboratory Press, 1.76-1.81). 10 μ l of the ligation mixture were added to each aliquot of competent cells, defrosted on ice. After the cells had been incubated on ice for 30 minutes they were subjected to thermal shock at 42°C for 90 seconds and were then replaced in ice for 60 seconds. After the addition of 0.5 ml of SOC broth (2% Bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 20 mM glucose, pH 7), the cells were incubated at 37°C with stirring for 90'. 100, 200 and 300 μ l aliquots were spread on plates containing solid LB broth (10 gr/l NaCl, 10 gr/l Bactotryptone, 5 gr/l yeast extract, pH 7.5, 15 gr/l agar) with the addition of 50 μ g/ml of ampicillin and in the presence of IPTG and X-Gal. The plates were then incubated at 37°C overnight.

Some of the bacterial colonies obtained were first analyzed for their plasmid content by a quick method (Maniatis, Molecular cloning, 1989, Cold Spring Harbor Laboratory Press, 1.32). The colonies containing a plasmid of the expected length were grown and their plasmid DNA extracted (Maniatis, Molecular cloning, 1989, Cold Spring Harbor Laboratory Press, 1.33). Those containing a fragment of the expected length (1160 bp) were identified by digestion of the plasmid DNA with Eco RI. The E1 colony was selected.

One end of the insert of the E1 colony was sequenced. The plasmid DNA of the E1 clone was denatured and partially sequenced by Sanger's method using the enzyme Sequenase and ³⁵S-dATP (Amersham). The sequencing products were separated on 8% acrylamide, 8 M urea, 1XTBE gel. After electrophoresis, the gel was dried and exposed overnight in contact with an autoradiographic plate (β max, Amersham). The sequence was compared with that published and was identical, identifying the *Arabidopsis* FAD2 gene in the cloned fragment.

45 Extraction of nucleic acids from hazel

Hazelnuts of the Nocchione, Montebello and San Giovanni varieties were harvested when almost fully ripe. The kernel was skinned before being used or frozen in liquid nitrogen. The leaves were harvested at a young stage and frozen in liquid nitrogen. 3 ml of extraction buffer were used for each gram of vegetable material with the use of the method described by Verwoerd et al. (Nucl. Ac. Res., 1989, 2362). Upon completion of the extraction, two selective precipitations were carried out by the addition of NaCl 2 M, and 2 volumes of 95% ethanol to eliminate polysaccharides. The final pellet was resuspended in H₂O. Further centrifuging was then carried out to eliminate any non-resuspended material.

On the other hand, DNA was extracted from young leaves of the Nocchione and Montebello varieties. The vegetable tissue was pulverized in liquid nitrogen and the DNA extracted by the CTAB (REF) method. To eliminate the polysaccharides, NaCl 2 M and 2 volumes of 95% ethanol were added. The samples were incubated for 15' at -80°C and centrifuged for 15' at 4°C and 14000 rpm (Eppendorf). This selective precipitation was repeated twice and the final pellet was resuspended in H₂O. Further centrifuging was then carried out to eliminate any non-resuspended material.

Checking of the probe on hazel DNA and RNA

About 20 µg of DNA of the Montebello and Nocchione varieties was cut with Eco RI restriction enzyme in a volume of 300 µl in the presence of 400 units of enzyme and H buffer (Promega), with incubation for one night at 37°C. After digestion had been checked by gel electrophoresis of one twentieth of the reaction mixture, the samples were precipitated with ethanol and resuspended in 30 µl of H₂O. The DNA was then subjected to electrophoresis on 0.7% agarose gel and transferred by capillarity onto nylon membrane (Southern blot) for one night in the presence of 20 x SSC (3M NaCl, 0.3M Na citrate). The membrane was dried in air for 30' and then fixed by UV treatment (120,000 µJ/cm²).

The *Arabidopsis* Δ12 desaturase gene was used as a probe. For this purpose, the plasmid DNA of the E1 clone (5 µg) was cut with 20 units of Eco RI in the presence of H buffer (Boehringer) in a volume of 30 µl for 12 hours at 37°C. The insert of the clone was separated from the vector by electrophoresis on 1% agarose gel and extracted from the gel with the use of Qiaex resin in accordance with the suppliers' instructions (Qiagen). The DNA was denatured for 10' at 100°C, cooled rapidly in dry ice, and marked by the random priming method with the use of 6000 Ci/mmol (α³²P) dATP and the reagents of Boehringer's marking kit.

The nylon membrane containing the hazel DNA was prehybridized for 1.5 hours at 55°C in standard buffer (5 x SSC, 0.1% (w/v) N-laurylsarcosine, 0.02% SDS, 1% blocking reagent solution) (10% blocking reagent solution: 10gr Boehringer blocking reagent in 150mM NaCl, 100mM maleic acid, pH7.5). The membrane was then hybridized with the *Arabidopsis* probe for one night at 55°C. The non-hybridized probe was washed twice for 15' in 2 x SSC, 0.1% SDS and twice for 15' each in 0.3 x SSC, 0.1% SDS, always at a temperature of 55°C. The probe remained coupled to the homologous sequences on the membrane was detected by autoradiography.

The RNA extracted from the young leaves of the Montebello and Nocchione varieties and from the kernels of the San Giovanni variety was separated on denaturing gel in the presence of formamide and transferred to nylon membrane by Northern blotting (Maniatis, Molecular cloning, 1989, Cold Spring Harbor Laboratory Press, 7.43-7.45). 40 µg/sample of total RNA extracted from San Giovanni kernels, Nocchione leaves and Montebello leaves were used. 60 pg of probe were used as a positive control. The RNA was loaded onto a 1% agarose gel in the presence of formaldehyde. The samples were then subjected to electrophoresis for 3 hours at 80 volts in the presence of 1xMOPS. The gel was rinsed in H₂O and then stained with ethidium bromide 0.5 µg/ml to display the RNA. The RNA was then transferred onto a nylon membrane (Boehringer) by "capillary blotting" in the presence of 20 x SSC throughout the night at 4°C. After transfer, the membrane was dried on 3 MM paper and then fixed by crosslinking using UV light (Stratagene UV Stratalinker 120000 µJ/cm²). The RNA was hybridised with the *Arabidopsis* Δ12 desaturase probe as described for the DNA. Detection was carried out by autoradiography. The heterologous *Arabidopsis* probe was able to display a band with a molecular weight of about 1500 bp in the hazel RNA and 3 bands of about 18, 8 and 2.8 kb in the hazel DNA cut with Eco RI.

Construction of a gene library of cDNA

The gene library of cDNA was constructed from RNA from kernels harvested when almost fully ripe and taken from plants of the San Giovanni variety. For this purpose, the Poly(A)+mRNA was isolated from the total RNA with the use of the Poly(A)Tract mRNA Isolation System II, in accordance with the method provided by the firm Promega. The samples were eluted in H₂O and precipitated with 0.1 volumes of 3M NaOAc and 3 volumes of 95% ethanol. After one night at -80°C, the RNA was centrifuged for 15' at 14000 rpm (Eppendorf), the pellet was rinsed in 75% ethanol and resuspended in 10 µl of H₂O. The concentration was read with a spectrophotometer and the yield was 3.2 µg of Poly(A)+mRNA per mg of total RNA.

The messenger RNA polyadenilate derived from kernels of the San Giovanni variety was used as a template for the synthesis of complementary DNA (cDNA) with the use of Boehringer's "cDNA synthesis kit" in accordance with the method recommended by the suppliers. An extraction was then carried out with one volume of phenol:chloroform: isoamyl alcohol (25:24:1). The cDNA was then purified in a Pharmacia column (cDNA spun columns) after the addition of NaCl 100 mM. The buffer used was the following: 10mM Tris-HCl pH 7.5, 1mM EDTA, 150mM NaCl. Eco RI "adaptors" (Pharmacia) were added to the ends of the cDNA. The reaction mixture contained: 5 µl of cDNA (half of the cDNA obtained from 6 µg of Poly(A)+RNA), 10 µl of ligase buffer 10 x (Promega), 10 µl of Eco RI adaptors (0.01 u/µl), 6 units of T4 DNA ligase (Promega), in a final volume of 100 µl. After incubation for 12 hours at 12°C, the ligase enzyme was inactivated for 10' at 65°C. Phosphorylation of the adaptors then followed by the addition, to the 100 µl mixture, of 10 µl of 100mM ATP and 10 units of T4 polynucleotide kinase. After incubation at 37°C for 30', the enzyme was inactivated by incubation for 10' at 65°C. Purification was then carried out with one volume of phenol:chloroform:isoamyl alcohol (25:24:1). The cDNA was then purified from fragments of less than 400 bp as follows. After the addition of NaCl to a final concentration of 0.1M NaCl, the cDNA was separated by chromatography in a column with Sepharose CL-4B resin (Size prep 400 spun column, Pharmacia) according to the method suggested by the suppliers. The fragments of cDNA shorter than 400 bases were thus excluded. The cDNA was precipitated with one thirtieth of a volume of 3M NaOAc and 2 volumes of 95% ethanol, centrifuged and resuspended in 10 µl of H₂O.

The cDNA was inserted in the λ phage vector Zap II cut with Eco RI and dephosphorylated (Stratagene) in the following manner: 2 μ l of cDNA (200 ng), 1 μ l of λ Zap II cut with Eco RI (1 μ g/ μ l) (Stratagene), 0.5 μ l of T4 DNA Ligase (4U/ μ l) (Promega), 0.5 μ l of 10 x ligation buffer (Promega), 1 μ l of H₂O. The reaction mixture was incubated for 14 hours at 12°C. The mixture containing the cDNA inserted in the vector was used for the reconstruction of the phages with the use of Stratagene's Gigapack Gold "in vitro packaging" kit. The gene library of phages thus obtained was constituted by about 300,000 pfu (plaque-forming units). In order to amplify the gene library, XL1 Blue MRF' cells were prepared as described by Stratagene and used the same day. The gene libraries were plated at a concentration of about 5000 pfu per plate (95 cm²). After growth, the phages were resuspended in SM (5.8gr/l NaCl, 2gr/l MgSO₄·7H₂O, 50ml/l 1M Tris HCl (pH 7.5), 5ml/l 2% gelatine) and, after the addition of chloroform to 5% and incubation for 15 minutes at ambient temperature, the cell debris was centrifuged for 10 minutes at 2000 x g. Chloroform to 0.3% was added to the supernatant liquid and the phages were preserved at 4°C. Aliquots were preserved at -70°C after the addition of DMSO to 7%. The gene library was titled.

Construction of a partial genome gene library

The DNA of the Nocchione variety was digested with Eco RI restriction enzyme and separated on agarose gel. The fragments with lengths of up to 10000 bp (base pairs) were isolated from the gel with the use of Qiaex resin according to the Qiagen's method. For cloning in the λ vector Zap II, 400ng of DNA fragments were incubated with 1 μ g of desphosphorylated λ Zap II (Stratagene) in the presence of ligase buffer and 1.5 units of T4 DNA ligase (Promega) for 12 hours at 14°C.

Stratagene's Gigapack Gold "in vitro packaging" kit was used in accordance with the suppliers' instructions to make up the gene library. The gene library of phages thus produced was amplified as described for the cDNA gene library. The complexity of the gene library was 1,500,000 clones. This gene library was also amplified.

Screening of the cDNA gene library

About 250,000 phages of the cDNA gene library were plated on LB broth in the presence of XL1 Blue MRF' cells, divided into 12 plates each containing 20,000 pfu. After growth, the phages were transferred onto nylon membranes and their denatured DNA was fixed on the membranes as described by Boehringer for screening with non-radioactive probes. The membranes were then hybridized with the *Arabidopsis* Δ 12 desaturase gene. The probe was prepared by the isolation of the insert containing the entire coding region of the gene from the plasmid. The insert was then marked with digoxigenin-dUTP with the use of Boehringer's "DNA labelling kit". Prehybridization was carried out in standard buffer (Boehringer) and hybridization was carried out in the same buffer with the addition of the *Arabidopsis* probe at a concentration of 10ng/ml and at a temperature of 55°C.

After washing twice in 2xSSC, 0.1% SDS for 5 minutes at ambient temperature and washing twice in 0.3xSSC, 0.1%SDS at 55°C, detection was carried out with the use of an anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer) and a chemiluminescent substrate (AMPPD, Boehringer).

11 positive phage plaques were identified. These were isolated, the phages resuspended in SM and titled. From 50 to 200 phages were plated for each positive plaque. The plaques were transferred onto nylon membranes and subjected to a second hybridization with the *Arabidopsis* Δ 12 desaturase probe, as already described above. The following clones which could hybridize with the *Acabidopsis* Δ 12 desaturase gene were obtained from the second screening: I, F, 4.

Screening of the genome gene library

The gene library of Nocchione DNA was subjected to screening in the same way as the cDNA gene library. 1,600,000 phages were plated, divided into 40 plates. After growth, they were transferred to nylon membranes as described for the cDNA gene library. The membranes were then hybridized with the *Arabidopsis* Δ 12 desaturase gene as described for the cDNA gene library. Autoradiography of the membranes showed 9 positive plaques. These plaques were isolated, titled and subjected to a second screening. 6 plaques were re-confirmed as positive. 4 of these gave a very strong signal.

Analysis of the clones isolated

The following positive phage clones were converted into plasmids by *in vivo* excision in accordance with the method suggested by Stratagene (Gigapack Gold in vitro packaging) : I, F, 4 (cDNA gene library), N2, N11, N17, N18, N21, N25 (genome gene library).

The plasmid DNA of the clones of the cDNA gene library was isolated and the length of the insert analyzed by digestion with Eco RI. The plasmid DNA of the genome clones was isolated, the length of the insert analyzed by cutting

with restriction enzyme, and the clones rechecked by hybridization with the *Arabidopsis* probe. Figure 1 shows the map of the N2 genome clone.

Sequencing

The N2 clone was selected from the genome clones. For sequencing, the insert was fragmented with Sau3A restriction enzyme and the fragments obtained were subcloned in pUC18 vector cut with BamHI (Maniatis, Molecular cloning, 1989, Cold Spring Harbor Laboratory Press, 1.68-1.69). The clones obtained were analyzed both for the length of the insert and by hybridization with the *Arabidopsis* probe. Since the N2 insert was 2.8 kb and hence longer than the $\Delta 12$ desaturase gene, the hybridization excluded the clones containing sequences outside the gene. The insert of the I, F, 4 and N2 clones was isolated and sequenced with the use of the Sequenase kit and (35S)dATP. All of the clones (cDNA and genome) were first sequenced at the ends with the use of primers which could couple with the vector in both orientations. In order to complete the internal regions and to assemble the fragments of the N2 genome clone, internal oligonucleotides were then designed and synthesized and were used for the sequencing. The following table shows the sequences of the internal oligonucleotides:

OLIGONUCLEOTIDE	SEQUENCE
N2-3SS	CAG ACC AGC ATC CGA GAC
N2-3SD	GGA TTG GCT TAG GGG GGC
N2-29R'S	GCC AAC CAT GTC ATC AAC CC
NOCCS	ATG GTA GAG AAG AGA TGG TG
COL	CTG GTG GGT TGT TGA AG
N2-S1N	GGA GAG GTC ATA AAC AAC

The I and F clones were sequenced entirely. As far as the N2 clone is concerned, only the regions corresponding to the gene were sequenced. Figures 2 and 3 show their sequence. The I and F cDNA clones were identical. A comparison between I and the N2 genome clone showed the same sequence (Fig. 4), indicating that N2 contains the gene which codes for the cDNA of the I clone.

Comparison between the gene isolated and other desaturases

The nucleotide and amino-acid sequence of the N2 clone was compared with other desaturases (Figure 6). The greatest homology was with the two $\Delta 12$ desaturases of the endoplasmic reticulum and with a hydroxylase of ricin which uses the same substrate as $\Delta 12$ desaturase. Homology with the plastid $\Delta 12$ desaturases and with both the plastid and endoplasmic reticulum $\Delta 15$ desaturases was, however, much lower. Figure 5 shows the comparison between the amino-acid sequence of hazel $\Delta 12$ and those of *Arabidopsis* and soya.

Checking of the expression of the hazel $\Delta 12$ desaturase gene

RNA was extracted from kernels of the San Giovanni, Montebello and Nocchione varieties and from leaves of the Montebello and Nocchione varieties. After separation on agarose gel, the RNA was transferred onto a nylon membrane and hybridized with the insert of the I clone marked with digoxigenin. The result is shown in Figure 7, in which a band is visible in the kernel RNA but not in that of the leaves.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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- (E) COUNTRY: Belgium
- (F) POSTAL CODE (ZIP): 6700

(ii) TITLE OF INVENTION: Isolation and sequencing of the
hazel FAD2-N
gene

(iii) NUMBER OF SEQUENCES: 4

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(EPO)

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: CH 0550/96
- (B) FILING DATE: 04-MAR-1996

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1662 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Corylus avellana cv. Nocchione
- (F) TISSUE TYPE: leaves

(vii) IMMEDIATE SOURCE:

- (B) CLONE: N2

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 222..1370
- (D) OTHER INFORMATION: /product= "delta-12 desaturase"
/gene= "Fad2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CCTCATAAAA AAGTAAGCTC ATTTACCTCA AGTAGGGTTT CCTTATGACA AATGAGTCCC
60

GCAATCCTTT TCTATGAGGT GCTATAATTG CAAATGTCCA AATCATAGGG ATATGGATCC
120

AAATACTATT AATATTATGT AGTGTGTTTT TTTTTTCCC TCAAATTTAC TCTCACACCT
180

AAGTTGATTT TCTCCAGCAT TGGACATAGC CTCTGTAGAC A ATG GGA GCT AGA
233

Met Gly Ala Arg
1

AGC CGA ATG CCT GCT ACC AAC AAG CCT AAA GAG CAA AAA ACA CCC ATC
281

Ser Arg Met Pro Ala Thr Asn Lys Pro Lys Glu Gln Lys Thr Pro Ile

5 10 15 20

CAG CGA GCA CCA CAC ACA AAA CCC CCA TTC ACT CTT AGC CAA CTC AAG
329

Gln Arg Ala Pro His Thr Lys Pro Pro Phe Thr Leu Ser Gln Leu Lys

25 30 35

AAA GCC GTC CCA CCC AAT TGT TTC CAA CGC TCT CTC CTA CGC TCG TTC
377

Lys Ala Val Pro Pro Asn Cys Phe Gln Arg Ser Leu Leu Arg Ser Phe

40 45 50

TCA TAT GTT GTT TAT GAC CTC TCC TTA GCC TTC CTC TTC TAC TAT ATT
425

Ser Tyr Val Val Tyr Asp Leu Ser Leu Ala Phe Leu Phe Tyr Tyr Ile

55 60 65

GCT ACC TCT TAC TTC CAT CTC CTC CCT CAC CCC CTT TCC TAC TTG GCA
473

Ala Thr Ser Tyr Phe His Leu Leu Pro His Pro Leu Ser Tyr Leu Ala

70 75 80

TGG TCA ATC TAT TGG GCT CTC CAA GGC TGC ATT CTC ACC GGC GTT TGG
521

Trp Ser Ile Tyr Trp Ala Leu Gln Gly Cys Ile Leu Thr Gly Val Trp

85 90 95 100

EP 0 794 250 A1

5 GTC ATC GCA CAT GAG TGC GGT CAC CAT GCC TTT AGT GAC TAC CAA TGG
 569
 Val Ile Ala His Glu Cys Gly His His Ala Phe Ser Asp Tyr Gln Trp
 105 110 115

 10 GTT GAT GAC ATG GTT GGC CTA ACC CTT CAC TCT GCT CTT TTA GTT CCA
 617
 Val Asp Asp Met Val Gly Leu Thr Leu His Ser Ala Leu Leu Val Pro
 120 125 130

 15 TAC TTT TCA TGG AAG ATT AGC CAC TGT CGC CAC CAC TCT AAC ACC GGC
 665
 Tyr Phe Ser Trp Lys Ile Ser His Cys Arg His His Ser Asn Thr Gly
 135 140 145

 20 TCC CTT GAC CGA GAT GAG GTG TTT GTC CCC AAG CCG AAA TCC AAA ATG
 713
 Ser Leu Asp Arg Asp Glu Val Phe Val Pro Lys Pro Lys Ser Lys Met
 150 155 160
 25

 30 CCA TGG TTT TCT AAG TAC TTC AAC AAC CCA CCA GGT AGG GTC CTC ACT
 761
 Pro Trp Phe Ser Lys Tyr Phe Asn Asn Pro Pro Gly Arg Val Leu Thr
 165 170 175 180

 35 CTT TTG ATC ACA CTC ACT CTA GGC TGG CCC TTG TAC TTA GCC TTG AAT
 809
 Leu Leu Ile Thr Leu Thr Leu Gly Trp Pro Leu Tyr Leu Ala Leu Asn
 185 190 195

 40 GTT TCT GGC CGA CCC TAT GAT CGT TTT GCT TGC CAC TAT GAT CCC TAT
 857
 Val Ser Gly Arg Pro Tyr Asp Arg Phe Ala Cys His Tyr Asp Pro Tyr
 200 205 210

 45 GGC CCC ATT TAT TCC AAT CGC GAA AGG TGT CAA ATA TTT GTC TCG GAT
 905
 Gly Pro Ile Tyr Ser Asn Arg Glu Arg Cys Gln Ile Phe Val Ser Asp
 215 220 225
 50

 55 GCT GGT GTC TTT GCT ACA ACT TAT GTG CTT TAC TAC GCA GCA ATG TCA
 953

EP 0 794 250 A1

Ala Gly Val Phe Ala Thr Thr Tyr Val Leu Tyr Tyr Ala Ala Met Ser
230 235 240

5

AAA GGG CTG GCA TGG CTT GTA TTC ATT TAT GGT ATG CCA TTG CTC ATA
1001
Lys Gly Leu Ala Trp Leu Val Phe Ile Tyr Gly Met Pro Leu Leu Ile

10

245 250 255 260

GTG AAT GGC TTC CTT GTA TTA ATC ACC TAC TTG CAG CAC ACT CAC CCT
1049

15

Val Asn Gly Phe Leu Val Leu Ile Thr Tyr Leu Gln His Thr His Pro
265 270 275

GCA TTG CCG CAC TAT GAC TCA TCA GAA TGG GAT TGG CTT AGG GGG GCA
1097

20

Ala Leu Pro His Tyr Asp Ser Ser Glu Trp Asp Trp Leu Arg Gly Ala
280 285 290

25

TTG GCG ACG GCG GAT AGA GAT TAC GGA ATG CTG AAT AAG GTT TTC CAC
1145

Leu Ala Thr Ala Asp Arg Asp Tyr Gly Met Leu Asn Lys Val Phe His
295 300 305

30

AAT ATC ATA GAC ACC CAT GTG GCT CAC CAT CTC TTC TCT ACC ATG CCT
1193

Asn Ile Ile Asp Thr His Val Ala His His Leu Phe Ser Thr Met Pro
310 315 320

35

CAT TAC CAT GCA ATG GAA GCC ACC AAA GCA ATC AAG TCA ATA TTG GGC
1241

40

His Tyr His Ala Met Glu Ala Thr Lys Ala Ile Lys Ser Ile Leu Gly
325 330 335 340

AAA TAC TAC CAG TTT GAT GGC ACT CCA GTT TAC AAG GCA GTG TGG AGG
1289

45

Lys Tyr Tyr Gln Phe Asp Gly Thr Pro Val Tyr Lys Ala Val Trp Arg
345 350 355

50

GAG GCT AAA GAG TGC CTT TAT GTT GAG TCG GAC GAG GGG GCC CCT AAC
1337

Glu Ala Lys Glu Cys Leu Tyr Val Glu Ser Asp Glu Gly Ala Pro Asn

55

360 365 370

5 AAA GGT GTT TTC TGG TAT CAG AGC AAG CTG TGA TATTGGCTGG ATAGAGCCAA
1390
Lys Gly Val Phe Trp Tyr Gln Ser Lys Leu *

375 380

10 AGAAAATGTG ATTAGTAAGG TAGTGTCTTT GGTCAGTTTG GTGTGTTAAG GAACAAATAA
1450

15 TAATAATTAG CGACTATGAA TAGTTATTGT TAAACAAAAT TCACCCTTAT GTTTAGCAGG
1510

AACTTTTCTG GCTACACTTT TTTTCGTATG AAAAGCGCAT ATTTTTTAAT TGTTATATTG
1570

20 TTTTGACATT ACTCAAGCTT CAAAATTAAT ATCACAGAAA ATATCCAATG TCGAAGGTTT
1630

CATTGTAGGT TGAAAACTTT ATATTGAGGT GG
1662

25 (2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 383 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

35 Met Gly Ala Arg Ser Arg Met Pro Ala Thr Asn Lys Pro Lys Glu Gln
1 5 10 15

Lys Thr Pro Ile Gln Arg Ala Pro His Thr Lys Pro Pro Phe Thr Leu
20 25 30

40 Ser Gln Leu Lys Lys Ala Val Pro Pro Asn Cys Phe Gln Arg Ser Leu
35 40 45

45 Leu Arg Ser Phe Ser Tyr Val Val Tyr Asp Leu Ser Leu Ala Phe Leu
50 55 60

50 Phe Tyr Tyr Ile Ala Thr Ser Tyr Phe His Leu Leu Pro His Pro Leu
65 70 75 80

Ser Tyr Leu Ala Trp Ser Ile Tyr Trp Ala Leu Gln Gly Cys Ile Leu
85 90 95

55

EP 0 794 250 A1

Thr Gly Val Trp Val Ile Ala His Glu Cys Gly His His Ala Phe Ser
100 105 110

5 Asp Tyr Gln Trp Val Asp Asp Met Val Gly Leu Thr Leu His Ser Ala
115 120 125

10 Leu Leu Val Pro Tyr Phe Ser Trp Lys Ile Ser His Cys Arg His His
130 135 140

Ser Asn Thr Gly Ser Leu Asp Arg Asp Glu Val Phe Val Pro Lys Pro
145 150 155 160

15 Lys Ser Lys Met Pro Trp Phe Ser Lys Tyr Phe Asn Asn Pro Pro Gly
165 170 175

20 Arg Val Leu Thr Leu Leu Ile Thr Leu Thr Leu Gly Trp Pro Leu Tyr
180 185 190

Leu Ala Leu Asn Val Ser Gly Arg Pro Tyr Asp Arg Phe Ala Cys His
195 200 205

25 Tyr Asp Pro Tyr Gly Pro Ile Tyr Ser Asn Arg Glu Arg Cys Gln Ile
210 215 220

30 Phe Val Ser Asp Ala Gly Val Phe Ala Thr Thr Tyr Val Leu Tyr Tyr
225 230 235 240

Ala Ala Met Ser Lys Gly Leu Ala Trp Leu Val Phe Ile Tyr Gly Met
245 250 255

35 Pro Leu Leu Ile Val Asn Gly Phe Leu Val Leu Ile Thr Tyr Leu Gln
260 265 270

40 His Thr His Pro Ala Leu Pro His Tyr Asp Ser Ser Glu Trp Asp Trp
275 280 285

Leu Arg Gly Ala Leu Ala Thr Ala Asp Arg Asp Tyr Gly Met Leu Asn
290 295 300

45 Lys Val Phe His Asn Ile Ile Asp Thr His Val Ala His His Leu Phe
305 310 315 320

Ser Thr Met Pro His Tyr His Ala Met Glu Ala Thr Lys Ala Ile Lys
325 330 335

50 Ser Ile Leu Gly Lys Tyr Tyr Gln Phe Asp Gly Thr Pro Val Tyr Lys

55

EP 0 794 250 A1

95
 Ser Leu Ala Phe Leu Phe Tyr Tyr Ile Ala Thr Ser Tyr Phe His Leu
 5 400 405 410

 CTC CCT CAC CCC CTT TCC TAC TTG GCA TGG TCA ATC TAT TGG GCT CTC
 143
 10 Leu Pro His Pro Leu Ser Tyr Leu Ala Trp Ser Ile Tyr Trp Ala Leu
 415 420 425 430

 CAA GGC TGC ATT CTC ACC GGC GTT TGG GTC ATC GCA CAT GAG TGC GGT
 191
 15 Gln Gly Cys Ile Leu Thr Gly Val Trp Val Ile Ala His Glu Cys Gly
 435 440 445

 CAC CAT GCC TTT AGT GAC TAC CAA TGG GTT GAT GAC ATG GTT GGC CTA
 239
 20 His His Ala Phe Ser Asp Tyr Gln Trp Val Asp Asp Met Val Gly Leu
 450 455 460

 ACC CTT CAC TCT GCT CTT TTA GTT CCA TAC TTT TCA TGG AAG ATT AGC
 287
 25 Thr Leu His Ser Ala Leu Leu Val Pro Tyr Phe Ser Trp Lys Ile Ser
 465 470 475

 CAC TGT CGC CAC CAC TCT AAC ACC GGC TCC CTT GAC CGA GAT GAG GTG
 335
 35 His Cys Arg His His Ser Asn Thr Gly Ser Leu Asp Arg Asp Glu Val
 480 485 490

 TTT GTC CCC AAG CCG AAA TCC AAA ATG CCA TGG TTT TCT AAG TAC TTC
 383
 40 Phe Val Pro Lys Pro Lys Ser Lys Met Pro Trp Phe Ser Lys Tyr Phe
 495 500 505 510

 AAC AAC CCA CCA GGT AGG GTC CTC ACT CTT TTG ATC ACA CTC ACT CTA
 431
 45 Asn Asn Pro Pro Gly Arg Val Leu Thr Leu Leu Ile Thr Leu Thr Leu
 515 520 525

 GGC TGG CCC TTG TAC TTA GCC TTG AAT GTT TCT GGC CGA CCC TAT GAT
 479
 50 Gly Trp Pro Leu Tyr Leu Ala Leu Asn Val Ser Gly Arg Pro Tyr Asp

 55

EP 0 794 250 A1

	530	535	540
5	CGT TTT GCT TGC CAC TAT GAT CCC TAT GGC CCC ATT TAT TCC AAT CGC 527 Arg Phe Ala Cys His Tyr Asp Pro Tyr Gly Pro Ile Tyr Ser Asn Arg		
	545	550	555
10	GAA AGG TGT CAA ATA TTT GTC TCG GAT GCT GGT GTC TTT GCT ACA ACT 575 Glu Arg Cys Gln Ile Phe Val Ser Asp Ala Gly Val Phe Ala Thr Thr		
	560	565	570
15	TAT GTG CTT TAC TAC GCA GCA ATG TCA AAA GGG CTG GCA TGG CTT GTA 623 Tyr Val Leu Tyr Tyr Ala Ala Met Ser Lys Gly Leu Ala Trp Leu Val		
20	575	580	585 590
25	TTC ATT TAT GGT ATG CCA TTG CTC ATA GTG AAT GGC TTC CTT GTA TTA 671 Phe Ile Tyr Gly Met Pro Leu Leu Ile Val Asn Gly Phe Leu Val Leu		
	595	600	605
30	ATC ACC TAC TTG CAG CAC ACT CAC CCT GCA TTG CCG CAC TAT GAC TCA 719 Ile Thr Tyr Leu Gln His Thr His Pro Ala Leu Pro His Tyr Asp Ser		
	610	615	620
35	TCA GAA TGG GAT TGG CTT AGG GGG GCA TTG GCG ACG GCG GAT AGA GAT 767 Ser Glu Trp Asp Trp Leu Arg Gly Ala Leu Ala Thr Ala Asp Arg Asp		
	625	630	635
40	TAC GGA ATG CTG AAT AAG GTT TTC CAC AAT ATC ATA GAC ACC CAT GTG 815 Tyr Gly Met Leu Asn Lys Val Phe His Asn Ile Ile Asp Thr His Val		
45	640	645	650
50	GCT CAC CAT CTC TTC TCT ACC ATG CCT CAT TAC CAT GCA ATG GAA GCC 863 Ala His His Leu Phe Ser Thr Met Pro His Tyr His Ala Met Glu Ala		
	655	660	665 670

55

EP 0 794 250 A1

ACC AAA GCA ATC AAG TCA ATA TTG GGC AAA TAC TAC CAG TTT GAT GGC
911
Thr Lys Ala Ile Lys Ser Ile Leu Gly Lys Tyr Tyr Gln Phe Asp Gly

5 675 680 685

ACT CCA GTT TAC AAG GCA GTG TGG AGG GAG GCT AAA GAG TGC CTT TAT
959
Thr Pro Val Tyr Lys Ala Val Trp Arg Glu Ala Lys Glu Cys Leu Tyr

10 690 695 700

GTT GAG TCG GAC GAG GGG GCC CCT AAC AAA GGT GTT TTC TGG TAT CAG
1007
Val Glu Ser Asp Glu Gly Ala Pro Asn Lys Gly Val Phe Trp Tyr Gln

15 705 710 715

AGC AAG CTG TGA TATTGGCTGG ATAGAGCCAA AGAAAATGTG ATTAGTAAGG
1059
Ser Lys Leu *

25 TAGTGTCCTTT GGTCAGTTTG GTGTGTTAAG GAACAAATAA TAATAATTAG CGACTATGAA
1119

TAGTTATTGT TAAA
1133

30

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 339 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

40

Gln Arg Ser Leu Leu Arg Ser Phe Ser Tyr Val Val Tyr Asp Leu Ser
1 5 10 15

45

Leu Ala Phe Leu Phe Tyr Tyr Ile Ala Thr Ser Tyr Phe His Leu Leu
20 25 30

Pro His Pro Leu Ser Tyr Leu Ala Trp Ser Ile Tyr Trp Ala Leu Gln
35 40 45

50

Gly Cys Ile Leu Thr Gly Val Trp Val Ile Ala His Glu Cys Gly His

55

EP 0 794 250 A1

	50	55	60
5	His Ala Phe Ser Asp Tyr Gln Trp Val Asp Asp Met Val Gly Leu Thr 65 70 75 80		
	Leu His Ser Ala Leu Leu Val Pro Tyr Phe Ser Trp Lys Ile Ser His 85 90 95		
10	Cys Arg His His Ser Asn Thr Gly Ser Leu Asp Arg Asp Glu Val Phe 100 105 110		
15	Val Pro Lys Pro Lys Ser Lys Met Pro Trp Phe Ser Lys Tyr Phe Asn 115 120 125		
	Asn Pro Pro Gly Arg Val Leu Thr Leu Leu Ile Thr Leu Thr Leu Gly 130 135 140		
20	Trp Pro Leu Tyr Leu Ala Leu Asn Val Ser Gly Arg Pro Tyr Asp Arg 145 150 155 160		
25	Phe Ala Cys His Tyr Asp Pro Tyr Gly Pro Ile Tyr Ser Asn Arg Glu 165 170 175		
	Arg Cys Gln Ile Phe Val Ser Asp Ala Gly Val Phe Ala Thr Thr Tyr 180 185 190		
30	Val Leu Tyr Tyr Ala Ala Met Ser Lys Gly Leu Ala Trp Leu Val Phe 195 200 205		
35	Ile Tyr Gly Met Pro Leu Leu Ile Val Asn Gly Phe Leu Val Leu Ile 210 215 220		
40	Thr Tyr Leu Gln His Thr His Pro Ala Leu Pro His Tyr Asp Ser Ser 225 230 235 240		
	Glu Trp Asp Trp Leu Arg Gly Ala Leu Ala Thr Ala Asp Arg Asp Tyr 245 250 255		
45	Gly Met Leu Asn Lys Val Phe His Asn Ile Ile Asp Thr His Val Ala 260 265 270		
	His His Leu Phe Ser Thr Met Pro His Tyr His Ala Met Glu Ala Thr 275 280 285		
50	Lys Ala Ile Lys Ser Ile Leu Gly Lys Tyr Tyr Gln Phe Asp Gly Thr 290 295 300		
55			

Pro Val Tyr Lys Ala Val Trp Arg Glu Ala Lys Glu Cys Leu Tyr Val
305 310 315 320

5 Glu Ser Asp Glu Gly Ala Pro Asn Lys Gly Val Phe Trp Tyr Gln Ser
325 330 335

10 Lys Leu *

15 Claims

1. A fragment of DNA from hazel (*Corylus avellana* L.) comprising the nucleotide sequence shown in Figure 2.
- 20 2. A DNA fragment comprising the nucleotide sequence shown in Figure 2 from base 222 to base 1367, which codes for the hazel Δ 12 desaturase enzyme of the endoplasmic reticulum or for a homologous sequence which can code for the same amino-acid sequence.
3. A nucleotide sequence coding for a protein or peptide having an amino-acid homology greater than or equal to 80% and preferably greater than 90% with the hazel Δ 12 desaturase enzyme of the endoplasmic reticulum of Claim 2
25 and having the function of the said enzyme.
4. A recombinant DNA sequence comprising a DNA sequence according to Claims 1, 2 and 3, or a portion of such a sequence, together with sequences regulating expression.
- 30 5. A recombinant DNA molecule comprising a cloning vector in which a DNA sequence according to any one of Claims 1, 2, 3 and 4 is inserted.
6. A DNA molecule according to Claim 5, in which the cloning vector is a plasmid or a phage.
- 35 7. A DNA molecule according to Claim 4 or Claim 5 having the restriction map shown in Figure 1.
8. A host organism including a recombinant DNA molecule according to any one of Claims 3 to 6.
9. A host organism according to Claim 8, selected from a vegetable cell, an animal cell, and a micro-organism.
- 40 10. A genetically modified organism capable of expressing the FAD2-N gene, having the amino-acid sequence shown in Figure 2 from bp 222 to bp 1367, portions of this gene, or this gene conjugated with other molecules and containing sequences which can inactivate endogenous genes.
- 45 11. A hazel Δ 12 desaturase enzyme of the endoplasmic reticulum having the amino-acid sequence shown in Figure 2 in substantially pure form.
12. A fusion polypeptide comprising the amino-acid sequence of the enzyme of Claim 11, in which the amino-acids additively connected thereto do not interfere with the desaturase activity or can easily be eliminated.
- 50 13. The use of the FAD2-N gene coding for the hazel Δ 12 desaturase enzyme of the endoplasmic reticulum or of portions thereof for the isolation of enzymes having the function of hazel desaturase or of the desaturase of another species.
- 55 14. The use of the nucleotide sequences of the FAD2-N gene shown in Figure 2 for the construction of expression systems which can alter the fatty-acid content in hazel.

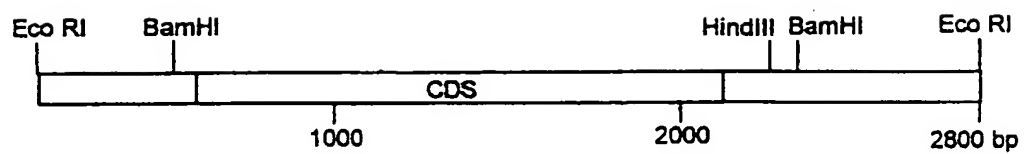


Fig. 1 - Restriction map of the genomic clone "N2". CDS: coding region; bp: base pair.

Fig. 2 - Nucleotide sequence of the gene FAD2-N corresponding to an internal fragment of the genomic clone "N2". Aminoacid residues of the coding region are also reported.

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CCTCATAAAAAAGTAAGCTCATTTACCTCAAGTAGGGTTTCCTTATGACAAATGAGTCCC 60
GGAGTAATTTTTTCATTCGAGTAAATGGAGTTCATCCCAAAGGAATACTGTTTACTCAGGG

GCAATCCTTTTCTATGAGGTGCTATAATTGCAAATGTCCAAATCATAGGGATATGGATCC 120
CGTTAGGAAAAGATACTCCACGATATTAACGTTTACAGGTTTAGTATCCCTATACCTAGG

AAATACTATTAATATTATGTAGTGTGTTTTTTTTTTTCCCTCAAATTTACTCTCACACCT 180
TTTATGATAATTATAATACATCACACAAAAAAGGGAGTTTAAATGAGAGTGTGGA

AAGTTGATTTTCTCCAGCATTGGACATAGCCTCTGTAGACAATGGGAGCTAGAAGCCGAA 240
TTCAACTAAAAGAGGTCGTAACTGTATCGGAGACATCTGTTACCCTCGATCTTCGGCTT
Met: Gly Ala Arg Ser Arg

TGCCTGCTACCAACAAGCCTAAAGAGCAAAAAACACCCATCCAGCGAGCACCACACACAA 300
ACGGACGATGGTTGTTTCGGATTCTCGTTTTTGTGGGTAGGTCGCTCGTGGTGTGTGTT
Met Pro Ala Thr Asn Lys Pro Lys Glu Gln Lys Thr Pro Ile Gln Arg Ala Pro His Thr

AAGCCCCATTCACTCTTAGCCAACTCAAGAAAGCCGTCCCAACCAATTGTTTCCAACGGT 360
TTGGGGTAAGTGAGAATCGGTTGAGTTCTTTGGGCAGGGTGGGTTAACAAAGGTTGCGA
Lys Pro Pro Phe Thr Leu Ser Gln Leu Lys Lys Ala Val Pro Pro Asn Cys Phe Gln Arg

CTCTCCTACGCTCGTTCTCATATGTTGTTTATGACCTCTCCTTAGCCTTCCTCTTCTACT 420
GAGAGGATGCGAGCAAGAGTATACAACAAATACTGGAGAGGAATCGGAAGGAGAAGATGA
Ser Leu Leu Arg Ser Phe Ser Tyr Val Val Tyr Asp Leu Ser Leu Ala Phe Leu Phe Tyr

ATATTGCTACCTCTTACTTCCATCTCCTCCCTCACCCCTTTCTTACTTGGCATGGTCAA 480
TATAACGATGGAGAATGAAGGTAGAGGAGGGAGTGGGGGAAAGGATGAACCGTACCAGTT
Tyr Ile Ala Thr Ser Tyr Phe His Leu Leu Pro His Pro Leu Ser Tyr Leu Ala Trp Ser

TCTATTGGGCTCTCCAAGGCTGCATTCTCACCGGCGTTTGGGTGATCGCACATGAGTGCG 540
AGATAACCCGAGAGGTTCCGACGTAAGAGTGCCGCAACCCAGTAGCGTGTAATCACGC
Ile Tyr Trp Ala Leu Gln Gly Cys Ile Leu Thr Gly Val Trp Val Ile Ala His Glu Cys

GTCACCATGCCTTTAGTGAATACTACCAATGGGTTGATGACATGGTTGGCCTAACCTTCACT 600
CAGTGGTACGGAATCACTGATGGTTACCAACTACTGTACCAACCGGATGGGAAGTGA
Gly His His Ala Phe Ser Asp Tyr Gln Trp Val Asp Asp Met Val Gly Leu Thr Leu His

CTGCTCTTTTAGTTCCATACTTTTCATGGAAGATTAGCCACTGTGCGCCACCACTCTAACA 660
GACGAGAAAATCAAGGTATGAAAAGTACCTTCTAATCGGTGACAGCGGTGGTGAGATTGT
Ser Ala Leu Leu Val Pro Tyr Phe Ser Trp Lys Ile Ser His Cys Arg His His Ser Asn

```

CCGGCTCCCTTGACCGAGATGAGGTGTTTGTCCCCAAGCCGAAATCCAAAATGCCATGGT 720
 GGCCGAGGGAACCTGGCTCTACTCCACAAACAGGGGTTTCGGCTTTAGGTTTTACGGTACCA
 Thr Gly Ser Leu Asp Arg Asp Glu Val Phe Val Pro Lys Pro Lys Ser Lys Met Pro Trp

TTTCTAAGTACTTCAACAACCCACCAGGTAGGGTCCTCACTCTTTTGATCACACTCACTC 780
 AAAGATTCATGAAGTTGTTGGGTGGTCCATCCCAGGAGTGAGAAAACCTAGTGTGAGTGAG
 Phe Ser Lys Tyr Phe Asn Asn Pro Pro Gly Arg Val Leu Thr Leu Leu Ile Thr Leu Thr

TAGGCTGGCCCTTGTTACTTAGCCTTGAATGTTTCTGGCCGACCCTATGATCGTTTTGCTT 840
 ATCCGACCGGGAACATGAATCGGAACCTACAAAGACCGGCTGGGATACTAGCAAAACGAA
 Leu Gly Trp Pro Leu Tyr Leu Ala Leu Asn Val Ser Gly Arg Pro Tyr Asp Arg Phe Ala

GCCACTATGATCCCTATGGCCCCATTTATTCCAATCGCGAAAGGTGTCAAATATTTGTCT 900
 CGGTGATACTAGGGATACCGGGGTAAATAAGGTTAGCGCTTTCCACAGTTTATAAACAGA
 Cys His Tyr Asp Pro Tyr Gly Pro Ile Tyr Ser Asn Arg Glu Arg Cys Gln Ile Phe Val

CGGATGCTGGTGTCTTTTGCTACAACCTTATGTGCTTTACTACGCAGCAATGTCAAAGGGC 960
 GCCTACGACCACAGAAACGATGTTGAATACACGAAATGATGCGTCGTACAGTTTTCCCG
 Ser Asp Ala Gly Val Phe Ala Thr Thr Tyr Val Leu Tyr Tyr Ala Ala Met Ser Lys Gly

TGGCATGGCTTGTTATTCATTTATGGTATGCCATTGCTCATAGTGAATGGCTTCCTTGTA 1020
 ACCGTACCGAACATAAGTAAATACCATACGGTAACGAGTATCACTTACCGAAGGAACATA
 Leu Ala Trp Leu Val Phe Ile Tyr Gly Met Pro Leu Leu Ile Val Asn Gly Phe Leu Val

TAATCACCTACTTGCAGCACACTCACCCCTGCATTGCCGCACTATGACTCATCAGAATGGG 1080
 ATTAETGGATGAACGTCGTGTGAGTGGGACGTAACGGCGTGATACTGAGTAGTCTTACCC
 Leu Ile Thr Tyr Leu Gln His Thr His Pro Ala Leu Pro His Tyr Asp Ser Ser Glu Trp

ATTGGCTTAGGGGGGCATTGGCGACGGCGGATAGAGATTACGGAATGCTGAATAAGGTTT 1140
 TAACCGAATCCCCCGTAACCGCTGCCGCCTATCTCTAATGCCTTACGACTTATTCCAAA
 Asp Trp Leu Arg Gly Ala Leu Ala Thr Ala Asp Arg Asp Tyr Gly Met Leu Asn Lys Val

TCCACAATATCATAGACACCCATGTGGCTCACCATCTCTTCTCTACCATGCCTCATTACC 1200
 AGGTGTTATAGTATCTGTGGGTACACCGAGTGGTAGAGAAGAGATGGTACGGAGTAATGG
 Phe His Asn Ile Ile Asp Thr His Val Ala His His Leu Phe Ser Thr Met Pro His Tyr

ATGCAATGGAAGCCACCAAAGCAATCAAGTCAATATTGGGCAAATACTACCAGTTTGATG 1260
 TACGTTACCTTCGGTGGTTTCGTTAGTTTACGTTATAACCCGTTTATGATGGTCAAACCTAC
 His Ala Met Glu Ala Thr Lys Ala Ile Lys Ser Ile Leu Gly Lys Tyr Tyr Gln Phe Asp

GCACTCCAGTTTACAAGGCAGTGTGGAGGGAGGCTAAAGAGTGCCTTTATGTTGAGTCGG 1320
 CGTGAGGTCAAATGTTCCGTACACCTCCCTCCGATTTCTCACGGAATACAACCTCAGCC
 Gly Thr Pro Val Tyr Lys Ala Val Trp Arg Glu Ala Lys Glu Cys Leu Tyr Val Glu Ser

ACGAGGGGGCCCCCTAACAAAGGTGTTTTCTGGTATCAGAGCAAGCTGTGATATTGGCTGG 138C
 TGCTCCCCCGGGGATTGTTTCCACAAAAGACCATAGTCTCGTTCGACACTATAACCGACC
 Asp Glu Gly Ala Pro Asn Lys Gly Val Phe Trp Tyr Gln Ser Lys Leu

ATAGAGCCAAAGAAAATGTGATTAGTAAGGTAGTGCTTTTGGTCAGTTTGGTGTGTTAAG 144C
 TATCTCGGTTTCTTTTACACTAATCATTCCATCACAGAAACCAGTCAAACCACACAATTC

GAACAAATAATAATAATTAGCGACTATGAATAGTTATTGTTAAACAAAATTCACCCCTTAT 150C
 CTTGTTTATTATTATTAATCGCTGATACTTATCAATAACAATTTGTTTTAAGTGGGAATA

GTTTAGCAGGAACTTTTCTGGCTACACTTTTTTTCGTATGAAAAGCGCATATTTTTTAAAT 156C
 CAAATCGTCCTTGAAAAGACCGATGTGAAAAAAGCATACTTTTCGCGTATAAAAAATTA

TGTTATATTGTTTTGACATTACTCAAGCTTCAAAATTAATATCACAGAAAATATCCAATG 162C
 ACAATATAACAAAACGTGAATGAGTTGGAAGTTTAAATTATAGTGCTTTTATAGGTTAC

TCGAAGGTTTCATTGTAGGTTGAAAACTTTATATTGAGGTGG 168C
 AGCTTCCAAAAGTAACATCCAACCTTTTGAAATATAACTCCACC

10 20 30 40 50
 tccaaagctctctctcctacgctcggttctcatatggtgtttatgacctctcc 50
 ttageccttccctcttctactatattgctacctcttacttccatctccccc 100
 tcaccccccttctctacttggcatggctccatctatagggtcttcccagggt 150
 gcattcttcccgccgttttgggtcctcgccacctgggtgggttcccatggc 200
 tttagtgactacccatgggttgatgacatgggtgggttcccccctccctc 250
 tgcctcttttagttccatctcttctcctggagcattggccactgtcgccccc 300
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 aactcccaaatgcccattgtttctcagtaacttccacccccccaggtag 400
 ggctccctccctcttttgcctcccccctactctcgggtggcccttgtacttag 450
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 cccctatggcccccatttcttccactcgccgacgggtgtcccatatttgcctc 550
 ggaatgctgggtgtctttgtctaccccttctgtgctttactcagcagccctgt 600
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 gtgaatggcttccctgtatttactcacttcttgcagcccccctcccccctgc 700
 attgcagccctctatgctcctcagcctgggaattggcttgggggggcaattgg 750
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 atagcccccctatgtgggtcaccatctcttctctaccatgcatcattcccc 850
 tgcattggagcgcaccccaagcattcagtcacatattgggccaatctctccc 900
 agtttgcctggccactccagtttccacggccgtgtggggggaggctccagag 950
 tgcatttatgttgggtcggacggggggccctcccccagggtgttttctg 1000
 gctacagagcagctgtgttatgtgctggctggctggcgcacccagccatgtga 1050
 ttagtaagggtagtgctcttgggtcagtttgggtgtgttaccggaccccatcct 1100
 acctattcgccgactatgcatcgttatgttccc 1133

Fig. 3 - Nucleotide sequence of cDNA clone "I".

Fig 4 - Nucleotide sequence alignment of clones "I" (I.SEQ) and "N2" (N2.SEQ).

```

1  ----- I.SEQ
1  CCTCATAAAAAAGTAAGCTCATTACCTCAAGTAGGGTTT N2.SEQ

1  ----- I.SEQ
41 CCTTATGACAAATGAGTCCCGCAATCCTTTTCTATGAGGT N2.SEQ

1  ----- I.SEQ
81 GCTATAATTGCAAAATGTCCAAATCATAGGGATATGGATCC N2.SEQ

1  ----- I.SEQ
121 AAATACTATTAAATATTATGTAGTGTGTTTTTTTTTCCC N2.SEQ

1  ----- I.SEQ
161 TCAAATTTACTCTCACACCTAAGTTGATTTTCTCCAGCAT N2.SEQ

1  ----- I.SEQ
201 TGGACATAGCCTCTGTAGACAAATGGGAGCTAGAAACCGAA N2.SEQ

1  ----- I.SEQ
241 TGGCTTGCTACCAACAAGCCTAAAGAGCAAAAAACACCCAT N2.SEQ

1  ----- I.SEQ
281 CCAGCGAGCACCAACACACAAAACCCCATTCACCTCTTAGC N2.SEQ

1  ----- I.SEQ
321 CAACTCAAGAAAGCCGTCCCAACCAATTGTTTCCAACGCT N2.SEQ

10 CTCTCCTACGCTCGTTCTCATATGTTGTTTATGACCTCTC I.SEQ
361 CTCTCCTACGCTCGTTCTCATATGTTGTTTATGACCTCTC N2.SEQ

36 CTTAGCCTTCCCTCTTCTACTATATTGCTACCTCTTACTTC I.SEQ
401 CTTAGCCTTCCCTCTTCTACTATATTGCTACCTCTTACTTC N2.SEQ

99 CATCTCCTCCCTCACCCCCCTTTCCTACTTGGCATGGTCAA I.SEQ
441 CATCTCCTCCCTCACCCCCCTTTCCTACTTGGCATGGTCAA N2.SEQ

130 TCTATTGGGGCTCTCCAAGGCTGCATTCTCACCGGGCGTTTG I.SEQ
481 TCTATTGGGGCTCTCCAAGGCTGCATTCTCACCGGGCGTTTG N2.SEQ

170 GGTTCATCGCACATGAGTGCGGTACCATGCTTTTAGTGAC I.SEQ
521 GGTTCATCGCACATGAGTGCGGTACCATGCTTTTAGTGAC N2.SEQ

210 TACCAATGGGTTGATGACATGGTTGGCCTAACCCCTTCACT I.SEQ
561 TACCAATGGGTTGATGACATGGTTGGCCTAACCCCTTCACT N2.SEQ

250 CTGCTCTTTTAGTTCCATACTTTTTCATGGAAGATTAGCCA I.SEQ
601 CTGCTCTTTTAGTTCCATACTTTTTCATGGAAGATTAGCCA N2.SEQ

290 CTGTCCCCACCACTCTAACACCGGCTCCCTTGACCGAGAT I.SEQ
641 CTGTCCCCACCACTCTAACACCGGCTCCCTTGACCGAGAT N2.SEQ

330 GAGGTGTTTGTCCCCAAGCCGAAATCCAAAATGCCATGGT I.SEQ
681 GAGGTGTTTGTCCCCAAGCCGAAATCCAAAATGCCATGGT N2.SEQ

370 TTTCTAAGTACTTCAACAACCCACCAAGGTAGGGTCTTCAC I.SEQ
721 TTTCTAAGTACTTCAACAACCCACCAAGGTAGGGTCTTCAC N2.SEQ

410 TCTTTTGATCACACTCACTCTAGGCTGGCCCTTGTACTTA I.SEQ
761 TCTTTTGATCACACTCACTCTAGGCTGGCCCTTGTACTTA N2.SEQ

450 GCCTTGAAATGTTTCTGGCCGACCCTATGATCGTTTTGCTT I.SEQ
801 GCCTTGAAATGTTTCTGGCCGACCCTATGATCGTTTTGCTT N2.SEQ

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490 GCCACTATGATCCCTATGGCCCCATTATTCCAATCGCGA I.SEQ
 841 GCCACTATGATCCCTATGGCCCCATTATTCCAATCGCGA N2.SEQ

 530 AAGGTGTCAAATATTTGTCTCGGATGCTGGTGTCTTTGCT I.SEQ
 881 AAGGTGTCAAATATTTGTCTCGGATGCTGGTGTCTTTGCT N2.SEQ

 570 ACAACTTATGTGCTTTACTACGCAGCAATGTCAAAAGGGC I.SEQ
 921 ACAACTTATGTGCTTTACTACGCAGCAATGTCAAAAGGGC N2.SEQ

 610 TGGCATGGCTTGTATTTCATTTATGGTATGCCATTGCTCAT I.SEQ
 961 TGGCATGGCTTGTATTTCATTTATGGTATGCCATTGCTCAT N2.SEQ

 650 AGTGAATGGCTTCCTTGTATTAATCACCTACTTGCAGCAC I.SEQ
 1001 AGTGAATGGCTTCCTTGTATTAATCACCTACTTGCAGCAC N2.SEQ

 690 ACTCACCTTGCATTGCCGCACTATGACTCATCAGAATGGG I.SEQ
 1041 ACTCACCTTGCATTGCCGCACTATGACTCATCAGAATGGG N2.SEQ

 730 ATTGCTTAGGGGGGGCATTGGCGACGGCGGATAGAGATTA I.SEQ
 1081 ATTGCTTAGGGGGGGCATTGGCGACGGCGGATAGAGATTA N2.SEQ

 770 CGGAATGCTGAATAAGGTTTTCCACAATATCATAGACACC I.SEQ
 1121 CGGAATGCTGAATAAGGTTTTCCACAATATCATAGACACC N2.SEQ

 810 CATGTGGCTCACCATCTCTTCTCTACCATGCCCTCATTAAC I.SEQ
 1161 CATGTGGCTCACCATCTCTTCTCTACCATGCCCTCATTAAC N2.SEQ

 850 ATGCAATGGAAGCCACCAAAGCAATCAAGTCAATATTGGG I.SEQ
 1201 ATGCAATGGAAGCCACCAAAGCAATCAAGTCAATATTGGG N2.SEQ

 890 CAAATACTACCAAGTTTGTATGGCACTCCAGTTTACAAGGCA I.SEQ
 1241 CAAATACTACCAAGTTTGTATGGCACTCCAGTTTACAAGGCA N2.SEQ

 930 GTGTGGAGGGAGGCTAAAGAGTGCCCTTTATGTTGAGTCGG I.SEQ
 1281 GTGTGGAGGGAGGCTAAAGAGTGCCCTTTATGTTGAGTCGG N2.SEQ

 970 ACGAGGGGGGCCCTAACAAGGTGTTTTCTGGTATCAGAG I.SEQ
 1321 ACGAGGGGGGCCCTAACAAGGTGTTTTCTGGTATCAGAG N2.SEQ

 1010 CAAGCTGTGATATTGGCTGGATAGAGCCAAAGAAAATGTG I.SEQ
 1361 CAAGCTGTGATATTGGCTGGATAGAGCCAAAGAAAATGTG N2.SEQ

 1050 ATTAGTAAGGTAGTGTCTTTGGTCAGTTTGGTGTGTTAAG I.SEQ
 1401 ATTAGTAAGGTAGTGTCTTTGGTCAGTTTGGTGTGTTAAG N2.SEQ

 1090 GAACAAATAATAATAATTAGCGACTATGAATAGTTATTGT I.SEQ
 1441 GAACAAATAATAATAATTAGCGACTATGAATAGTTATTGT N2.SEQ

 1130 TAAA I.SEQ
 1481 TAAACAAAATTCACCCCTTATGTTTAGCAGGAACCTTTCTG N2.SEQ

 1133 I.SEQ
 1521 GCTACACTTTTTTTTCGTATGAAAAGCGCATATTTTTTAAT N2.SEQ

 1133 I.SEQ
 1561 TGTATATATTGTTTTGACATTACTCAAGCTTCAAAATTAAT N2.SEQ

 1133 I.SEQ
 1601 ATCACAGAAAATATCCAATGTGGAAGGTTTCATTGTAGGT N2.SEQ

 1133 I.SEQ
 1641 TGAAAACCTTTATATTGAGGTGG N2.SEQ

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1  MGAIRSRMP - AITNKPKLEQRTPIQRAIPHTKPPFTLSQLKRAV N2.PRO
1  MGAGGRTDVPPANRKSEVDPLKRVPFEPQFSLSQIKRAI L43921.PRO
1  MGAGGRMPVPTSSSKKSEITDTTKRVPCIEKPPFSVGDILKRAI L26296.PRO

40  PPNCFQRS L LRSFSYVVYDLSLAFLEYIATSYFHL L P R P N2.PRO
41  PPBCFQRS V LRSFSYVVYDLTIAFCLEYVATHYFHL L P G P L43921.PRO
41  PPBCFQRS I PRSFSYLISDIIIASCFYVATNYFSL L P Q P L26296.PRO

80  LSYLAW S IYWA L QGCILTGVWVIAHECGHHA FSDYQWV D O N2.PRO
81  LSPRGM A IYWA V QGCILTGVWVIAHECGHHA FSDYQL L D O L43921.PRO
81  LSYLAW P LYWA C QGC V LTGIWVIAHECGHHA FSDYQWL D O L26296.PRO

120  MVGLTILHSALLVPYFSWKISRCHRHSNTGSLDERDEVFVPK N2.PRO
121  IVGLIILHSALLVPYFSWKYSRRRHSNTGSLERDEVFVPK L43921.PRO
121  TVGLIFHSFLLVPYFSWKYSRRRHSNTGSLERDEVFVPK L26296.PRO

160  PKSKMPWIFSKYFNNPPGRVLTLLITLTTLGWPLYLALNVS G N2.PRO
161  QKSCIRWYSKYLNPPGRVLTLLAVTTLGWPLYLALNVS G L43921.PRO
161  QKSAIKWYGYKYLNPNLGRIMMLTVQFVLGWPLYLAFNV S G L26296.PRO

200  RPYDRFACHYDPYGP IYSNRERCQIFVSDAGVFA TTYVLY N2.PRO
201  RPYDRFACHYDPYGP IYSNRERLQIYISDAGVLA VVYGLF L43921.PRO
201  RPYDGFACHFFFNAPIYNDRERLQIYLS DAGILA VC FGLY L26296.PRO

240  YAAMS KGLAWLVFIYGHPLLI VNGFLVLITYLQHTHPALP N2.PRO
241  RLAMAKGLAWVVCVYGVPLLVNGFLVLITFLQHTHPALP L43921.PRO
241  RYAAA AQGMA SMICLYGVPLLVNAFLVLITYLQHTHPSLP L26296.PRO

280  HYDSSEWDWLRGALATADR DYGMENKVFNHNIIDTHVAHHL N2.PRO
281  HYTSSSEWDWLRGALATVDR DYGILNKVFNHNIIDTHVAHHL L43921.PRO
281  HYDSSEWDWLRGALATVDR DYGILNKVFNHNIIDTHVAHHL L26296.PRO

320  FSTMPHYHAMEATKAIKSI L GKYYQFDGTPVYKAVWREAK N2.PRO
321  FSTMPHYHAMEATKAIKPI L GEYYRFOETPFVKAMWREAR L43921.PRO
321  FSTMPHYNAMEATKAIKPI L GOYYQFDGTPWYVAMYREAR L26296.PRO

360  ECLYVESDEGAPNKG VFWYQSKL N2.PRO
361  ECIYVEPDQSTESKGVFWYNNKL L43921.PRO
361  ECIYVEPDREGDKKGVYWYNNKL L26296.PRO

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Fig. 5 - Aminoacid sequence alignment of $\Delta 12$ desaturase from hazelnut (N2.PRO), *Arabidopsis* (L26296.PRO) and soybean (L43921.PRO). Homologous residues are boxed.

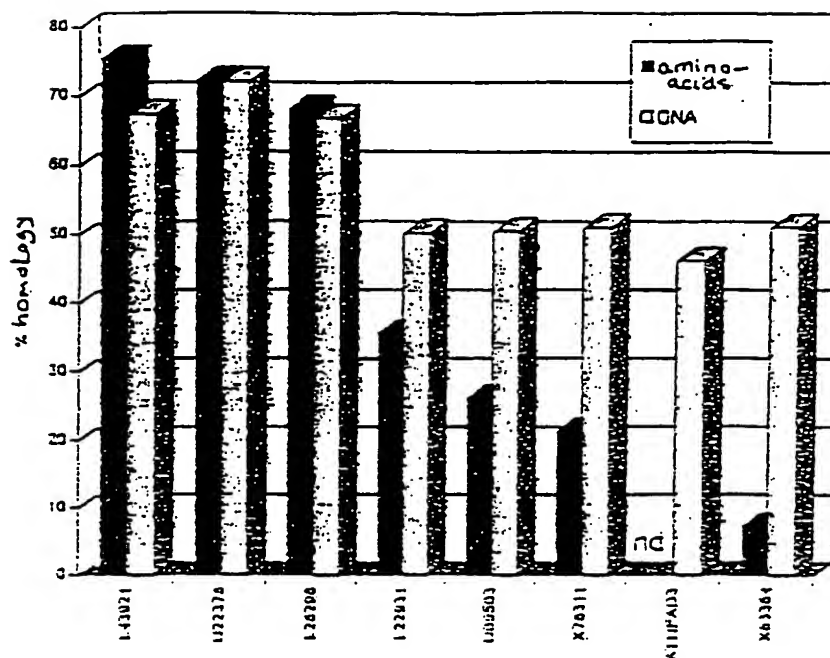


Fig. 6 - Homology between hazel $\Delta 12$ desaturase and other desaturases

- L43921: $\Delta 12$ desaturase of the endoplasmic reticulum of soya
 U22378: $\Delta 12$ hydroxylase of ricin
 L25296: $\Delta 12$ desaturase of the endoplasmic reticulum of *Arabidopsis thaliana*
 L22931: $\Delta 15$ plastid desaturase of *Arabidopsis thaliana*
 U09503: $\Delta 12$ plastid desaturase of *Arabidopsis thaliana*
 X78311: $\Delta 12$ plastid desaturase of spinach
 ATHFAD3: $\Delta 15$ desaturase of the endoplasmic reticulum of *Arabidopsis thaliana*
 X63364: $\Delta 9$ plastid desaturase of rape

Note: nd: not determined since the amino-acid sequence is not known.

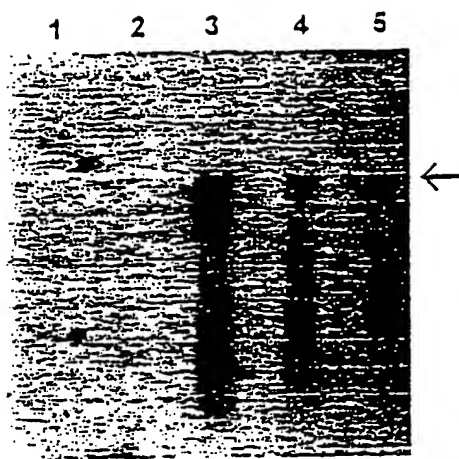


Fig. 7 - Northern blot of RNA of Montebello leaves (line 1), Nocchione leaves (line 2), Montebello kernels (line 3), Nocchione kernels (line 4), and San Giovanni kernels (line 5). The RNA was hybridized with the I clone of cDNA.



European Patent
Office

EUROPEAN SEARCH REPORT

Application Number
EP 97 10 3098

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X	WO 94 11516 A (DU PONT ;LIGHTNER JONATHAN EDWARD (US); OKULEY JOHN JOSEPH (US)) 26 May 1994 examples 1,6,7	10,13	C12N15/53 C12N15/82 C12N9/02 C12N5/10 C12Q1/68 //A01H5/00
A,D	THE PLANT CELL, vol. 6, January 1994, pages 147-158, XP002034147 OKULEY, J., ET AL . : "ARABIDOPSIS FAD2 GENE ENCODES THE ENZYME THAT IS ESSENTIAL FOR POLYSATURATED LIPID SYNTHESIS" * page 155, column 2, line 28 *	1-14	
A	WO 95 22598 A (DU PONT ;LETO KENNETH JOSEPH (US); ULRICH JAMES FRANCIS (US)) 24 August 1995 * page 10, line 1 *	1-23	
			TECHNICAL FIELDS SEARCHED (Int.Cl.6)
			C12N
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 3 July 1997	Examiner Holtorf, S
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons</p> <p>& : member of the same patent family, corresponding document</p>			

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